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Granulocyte Colony-Stimulating Factor Production by Human Bone Marrow Fibroblasts Stimulated With Interleukins

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Granulocyte colony-stimulating factor (G-CSF) is a cytokine that mediates the clonal proliferation and differentiation of progenitor cells into mature granulocytes. The kinetics of G-CSF production by human bone marrow fibroblasts (BMF) were investigated by quantitative immunoassays. The spontaneous production of G-CSF by BMF was below the detectable level. Interleukin-1 (IL-1) induced a dose-dependent production of G-CSF, and the production reached a plateau at 50 U/ml of IL-1. G-CSF production by BMF stimulated with IL-1 was cell concentration dependent. Detectable G-CSF was produced by 5×10^2 BMF in a 35 × 10-mm plastic dish. The optimal range was 1×10^4–5×10^4 BMF. Production of newly synthesized G-CSF was detectable 6 hr after stimulation and continued for approximately 48 hr. A 6-hr pulse exposure to IL-1 was necessary to induce production of G-CSF, and after 48 hr, the adherent BMF could not be restimulated. IL-2, IL-3, IL-4, IL-5, and IL-6 were unable to induce G-CSF production. However, IL-4 promoted G-CSF production after stimulation with IL-1. These results provide useful data with regard to the mechanism of G-CSF production by human BMF.

Key words: bone marrow, fibroblasts, G-CSF, IL-1, IL-4

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is a polypeptide growth factor that stimulates the production and function of granulocytes. These physiologic processes serve as the foundation for critical host defense systems and are maintained on a large scale in vivo [1]. Murine G-CSF has been purified to homogeneity and its cDNA clones have been identified [2]. Thereafter, the identity of human G-CSF was confirmed by sequence analysis [3], as well as by direct comparison with biological activity of recombinant G-CSF [4].

G-CSF is produced by various cells, including monocyte/macrophages [5,6]; fibroblasts [7]; vascular endothelial cells [8]; and neutrophils [9]; following appropriate stimulation. These cells are distributed widely in most tissues. G-CSF production is highly regulated but is not constitutive. Thus, it is well understood that local infections induce neutrophil production and enhancement of neutrophil function with G-CSF production by these cells. On the other hand, bone marrow stroma is considered to regulate normal granulopoiesis by producing G-CSF without exogenous stimulation. Hematopoiesis in bone marrow is considered to be clearly associated with stromal cells, which consist of fibroblasts, fat cells, endothelial cells, and macrophages. BMF are a chief constituent of bone marrow stromal cells. However, the physiologic control of G-CSF production by BMF remains incompletely understood. In particular, it is unclear what role is played by BMF in maintenance of normal steady-state granulopoiesis.

In order to better clarify the role of recombinant interleukins in supporting G-CSF production by BMF, we compared the abilities of interleukins-1, -2, -3, -4, -5, and -6 (IL-1, IL-2, IL-3, IL-4, IL-5, and IL-6), either alone or in various combinations, to stimulate G-CSF production from human BMF. Only IL-1 induced G-CSF production by BMF; the other interleukins did not induce

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G-CSF production. Moreover, only IL-4 promoted G-CSF production induced by IL-1.

MATERIALS AND METHODS

BMF Cultures

Bone marrow cells were obtained by aspiration from the sternum of consenting healthy volunteers, aged 20–40 years, with normal peripheral leukocyte counts and normal cell differential, hemoglobin, platelet counts, and cellular marrow on smear with a normal M:E ratio. The culture method of BMF was previously described [10]. Briefly, 10^7 viable nucleated cells were inoculated into 25-cm^2 tissue flasks containing 10 ml of supplemental α-medium (Gibco, Grand Island, NY), consisting of 20% fetal calf serum (FCS) (Gibco) and 1% antibiotic (Gibco). The cells were incubated in a 37°C, 5% CO_2-humidified environment. Following 20–26 hr of incubation, the supernatant and nonadherent cells were decanted, fresh medium was added, and the cultures were incubated for an additional 6 days. The cultures were fed weekly by replacement of one-half of the medium. When BMF formed a confluent monolayer (usually in 3–4 weeks), the cultures were passaged with 0.1% trypsin solution. A morphologically pure population of BMF was obtained after the third passage; subsequent experiments used these BMF. To confirm the morphological purity of these BMF, we performed the following immunoperoxidase studies.

Immunoperoxidase staining of membrane- and cytoplasm-associated components was carried out using the direct method [11]. BMF attached to glass coverslips were fixed with buffered paraformaldehyde solution for 30 sec and washed five times with phosphate-buffered saline (PBS).

The cells were incubated with polyclonal rabbit antiserum against collagen types I and III at room temperature for 30 min. Following three washes with PBS, the cell preparations were incubated with peroxidase-conjugated swine, antirabbit immunoglobulin at room temperature for 30 minutes, then washed five times with phosphate-buffered saline (PBS) and stained with diaminobenzidine.

The BMF monolayers showed extensive immunoperoxidase staining for type I collagen. This was observed in both cell cytoplasm and the fibrillar cytoplasmic extensions that formed a meshwork throughout the monolayers; staining for type III collagen was equally intense in the extracellular and intracellular spaces [10].

Results indicated that the cells growing at the bottom of the tissue flask were BMF and included few endothelial cells (BMF are thought to be differentiated from endothelial cells), and no adherent monocyte/macrophages.

Preparation of Conditioned Medium by BMF

After three to five passages in culture, adherent BMF were detached with trypsin. The cells were resuspended in 35 × 10-mm dishes (Iwaki Glass, Chiba, Japan) with 2 ml of α-medium containing 20% FCS. After 7 days of culture, the adherent cells were refed with fresh medium and were incubated in a fully humidified atmosphere with 5% CO_2 in air at 37°C, with various stimuli for specified time periods, as indicated under Results. The supernatant was removed after incubation and stored at −80°C until it was assayed for G-CSF.

The cytokines used as stimuli in these studies were recombinant human interleukin-1 (rhIL-1), rhIL-2, rhIL-3, rhIL-4, r mouse IL-5, and rhIL-6 (Genzyme, Cambridge, MA).

Assays for G-CSF

The concentration of G-CSF in each culture supernatant was determined [12,13] by a simple sequential sandwich enzyme immunoassay [11]. Briefly, the borate-buffered storage solution in anti-rG-CSF-coated tubes was removed by aspiration, and duplicate 0.2-ml aliquots of standards or unknowns were dispensed into the tubes. The samples were incubated for 2 hr at room temperature. Horseradish peroxidase (HPO)-labeled anti-rG-CSF rabbit Fab′ (0.1 ml) was then added, and the tubes were incubated for another 2 hr at room temperature. The tubes were washed three times, and 1 ml of substrate solution was added to each. After 1-hr incubation at room temperature in the dark, the reaction was stopped and absorbance values were read at 492 nm. The limit of sensitivity of the assay for G-CSF was 30 pg/ml.

Anti-rG-CSF Serum

Antiserum against rG-CSF was prepared in goats. The goats were immunized with a subcutaneous 2.5-mg injection of pure rG-CSF emulsified in an equal volume of Freund's complete adjuvant. Following the initial immunization, booster injections were given with 2.5 mg of purified antigen in the same adjuvant at 2-week intervals. On the fifth day after the last booster, blood was collected and stored at −20°C until use [14].

RESULTS

G-CSF Production by Bone Marrow Fibroblasts

Unstimulated resting adherent BMF did not produce any measurable amount of G-CSF. An initial screening of interleukins for stimulation of G-CSF production after 2-day incubation showed that only IL-1 induced G-CSF production from BMF (data not shown).

G-CSF production from BMF stimulated with IL-1 was cell concentration dependent (Fig. 1). G-CSF was detected from 5×10^2 BMF in a 35 × 10-mm plastic dish. The optimal range was $1–5 \times 10^4$ BMF.

IL-1 caused dose-dependent stimulation of G-CSF production. Titration of the dose of IL-1 indicated a linear relationship up to a dose of 50 U/ml, and maximal produc-

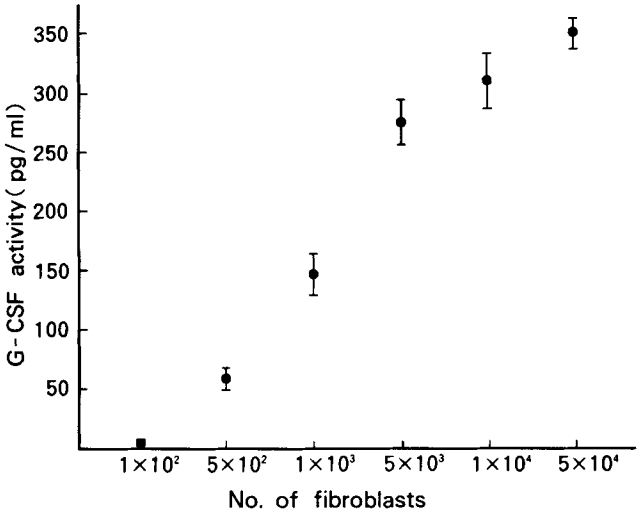

Fig. 1. G-CSF production by various numbers of fibroblasts after 48-hr stimulation with IL-1 50 U/ml. Results: mean ±SD of three experiments.

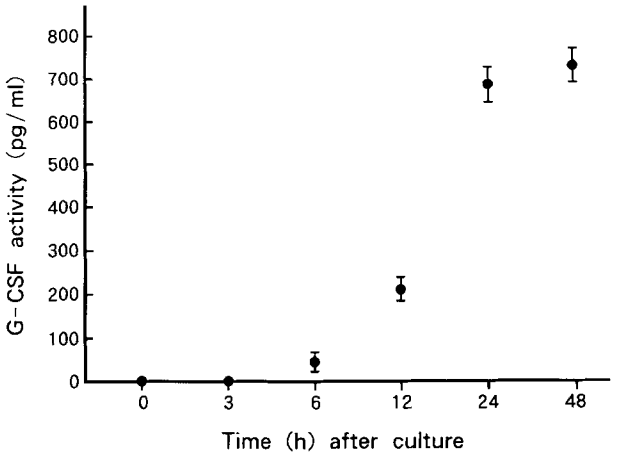

Fig. 3. G-CSF production by fibroblasts after continuous stimulation with IL-1 at 50 U/ml during 3, 6, 12, 24, and 48 hr. Results: mean ±SD in three experiments.

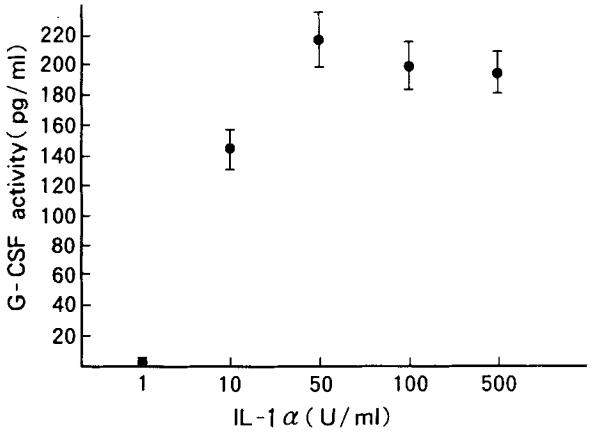

Fig. 2. G-CSF production by fibroblasts after 48-hr incubation with IL-1 at 1, 10, 50, 100, and 500 U/ml. Results: mean ±SD of three experiments.

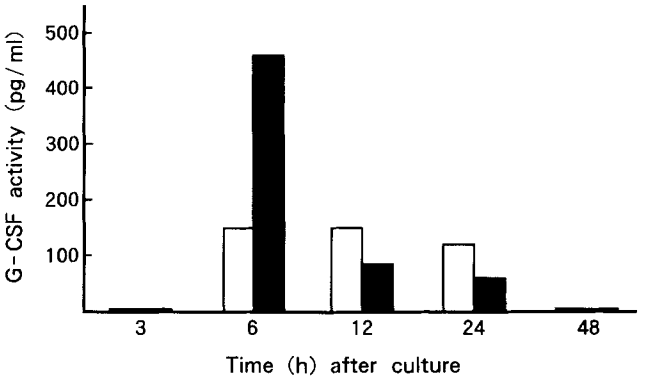

Fig. 4. G-CSF production by fibroblasts stimulated with IL-1 at 50 U/ml. At 3, 6, 12, 24, or 48 hr after stimulation, supernatant was removed and fresh medium with (■) or without (□) IL-1 50 U/ml was added. After 24-hr incubation, the supernatant was harvested for immunoassay.

tion was reached at 50–500 U/ml (Fig. 2). Preincubation with 20 μg of actinomycin D completely blocked G-CSF production by BMF stimulated with IL-1 (data not shown).

In addition, as a control stimulus, we tested the effects of various concentrations of phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, MO), concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO), phorbol 12-myristate 13-acetate (PMA) (Sigma), and lipopolysaccharide (LPS) (Sigma). Treatment with PHA, Con A, or PMA had no effect on BMF in any of the assays (data not shown). By contrast, LPS increased the level of G-CSF production by BMF, to a level similar to that obtained with IL-1α. A total of 20 ng/ml of LPS was required to induce maximum production of G-CSF; concentrations of ≤0.02 ng/ml had no effect (data not shown).

G-CSF production started rather slowly after stimulation with IL-1, and measurable amounts of G-CSF were usually detected in the supernatant within 6–12 hr (Fig. 3). Production continued in a linear fashion for 24 hr and then reached a plateau.

To determine whether the plateau in G-CSF production in the supernatant at 24–48 hr after IL-1 stimulation was due to consumption of the stimulus, or to exhaustion of G-CSF production by BMF, we removed the medium after 3, 6, 12, 24, and 48 hr and replaced it with a new dose of IL-1 (50 U/ml), followed by incubation for 24 hr. As shown in Figure 4, replacement of the medium and IL-1 at 6 hr resulted in continued G-CSF production, but the restimulating capacity had diminished by 24 hr and completely disappeared after 48 hr. On the other hand, when the medium was removed and replaced with

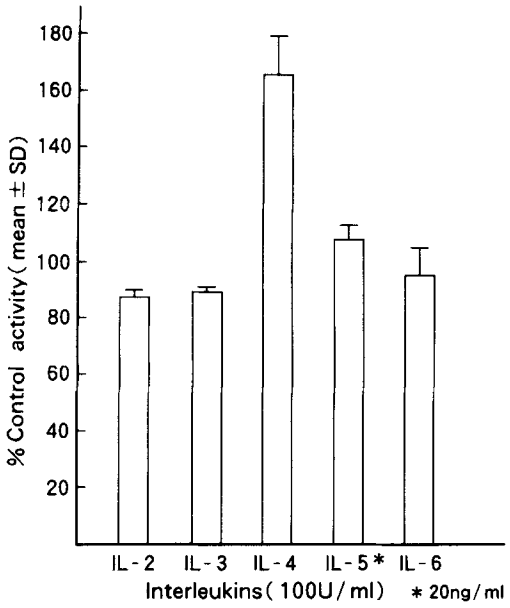

Fig. 5. Effects of various interleukins on G-CSF production by fibroblasts stimulated with IL-1 50 U/ml. The increase or reduction in G-CSF production is expressed as percentage of the production by fibroblasts stimulated with IL-1 50 U/ml alone. Results: mean ±SD in three experiments.

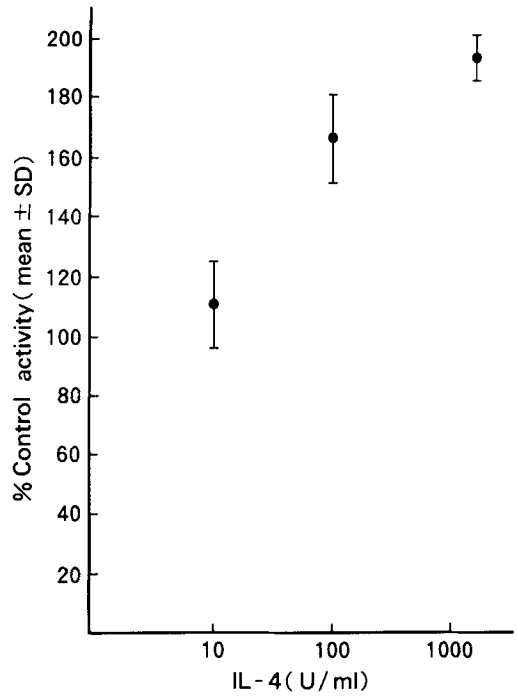

Fig. 6. Effects of IL-4 at 10, 100, and 1,000 U/ml on G-CSF production by fibroblasts stimulated with IL-1 50 U/ml for 48 hr. The increase in G-CSF production is expressed as % of the production by fibroblasts stimulated with IL-1 50 U/ml alone. Results: mean ±SD in three experiments.

fresh medium without IL-1 after 3, 6, 12, 24, and 48 hr of stimulation, significant G-CSF production could be measured after 24 hr of incubation, following 6–24 hr of stimulation. These experiments show that the first 6 hr of stimulation is necessary to induce G-CSF production and that the capacity of BMF to be stimulated decreases after approximately 24 hr. These observations showed that restimulation of BMF by IL-1 at 3 hr did not result in the production of G-CSF, but at 6 hr, a marked increase (approximately 10-fold) in G-CSF production was observed (Fig. 4), compared with the level observed after the initial stimulation (as shown in Fig. 3).

Effects of Different Interleukins on G-CSF Production

Having established that IL-1 stimulates G-CSF production by BMF, we conducted experiments to determine the effects of IL-2, IL-3, IL-4, IL-5, and IL-6 in combination with IL-1 on G-CSF production. After 2 days of culture, the activity of G-CSF in the supernatant was measured. As shown in Figure 5, statistical assessment of all groups revealed that only IL-4 significantly enhanced the effect of IL-1 ($P < 0.001$). Other interleukins neither promoted nor inhibited G-CSF production. IL-4 was tested at 10, 100, and 1,000 U/ml, but the maximal level of G-CSF production was not reached, even at the highest dose of IL-4 (Fig. 6).

DISCUSSION

In this study, a morphologically pure population of BMF was obtained after the third passage by using a liquid culture. The purity of these cells was estimated by immunoperoxidase staining with antitypes I and III collagen, and the cells demonstrated a fibroblastic nature [10].

In the past, the most commonly used methods for measuring G-CSF activity were colony-forming assays [12,13] or mRNA assays as Northern blots or in situ hybridization [14,15]. As Sallerfors and Olofsson [16] have shown, colony-forming assays are unspecific, since the tested samples contain various cytokines other than CSF, and the target bone marrow monocytes include cells with cytokine-producing capacity. The mRNA assays are specific but do not offer any information on the amounts of G-CSF produced. By contrast, immunoassays are specific and quantitative and, therefore, suitable as methods for measuring the production of G-CSF by BMF.

A few reports have been published in which immunoassays have been applied for quantification of G-CSF production by bone marrow stromal cells [17–20], synovial fibroblasts [21], chondrocytes [22], or monocytes [16,23]. However, no reports have been published concerning the production of G-CSF by a relatively pure population of human BMF. The adherent BMF investigated in this study

did not produce any significant amounts of G-CSF without exogenous stimuli. Only IL-1 induced G-CSF production in a dose-dependent manner. The blocking effect of actinomycin D and the discovery of an initial lag period of 6 hr before significant amounts of G-CSF are detected in the BMF supernatant indicate that G-CSF is newly synthesized and not secreted from preformed intracellular stores. It was also noted that G-CSF production by BMF lasts 24–48 hr and that after 48 hr the BMF could not be restimulated with IL-1 for further G-CSF production. These results suggest that BMF do not constitutively produce G-CSF without stimulation, but that they produce G-CSF for a definite time following appropriate stimulation. We also assayed IL-10 levels in the conditioned media (CM) of BMF (data not shown) in order to investigate the mechanism of shutdown of G-CSF production after 24–36 hr, which was found to be consistent with other models. The IL-10 levels were measured immunologically in the CM of BMF; then they increased (mean 53 pg/ml; range 7.3–581 pg/ml), so we suspect that this molecule was critical to the shutdown pathway of LPS-stimulated cells (detailed data not shown). Sallerfors and Olofsson [16] have clarified the kinetics of G-CSF secretion by human monocytes using quantitative immunoassays. IL-1 and LPS induced dose-dependent secretion of G-CSF. Secretion of newly synthesized G-CSF was detectable 3–6 hr after stimulation and continued for approximately 24 hr. Furthermore, a 20-min pulse exposure to LPS was sufficient to induce half-maximum secretion of CSF, and after 24–36 hr, the adherent monocytes could not be restimulated. These results show that the kinetics of G-CSF production by BMF in this study are quite similar to those of monocytes, although monocytes react to cytokines and produce G-CSF earlier than do BMF. These findings indicate that both BMF and monocytes play important roles in the regulation of granulopoiesis.

Using G-CSF production by BMF stimulated with IL-1, we did not find any evidence for either promotive or inhibitory effects of IL-2, IL-3, IL-5, and IL-6 on G-CSF production. Only IL-4 showed promotive effects, and these were dose dependent and twofold, in comparison with those of IL-1 alone. Tushinski et al. [24] have shown that, like IL-1, IL-4 can stimulate murine 3T3 fibroblasts to produce G-CSF, with a synergistic induction of the expression of high levels of G-CSF mRNA and protein in cells incubated in the presence of both IL-1 and IL-4. These investigators also found recently that IL-1 can stimulate normal human gingival fibroblasts to produce CSF, while IL-4 is unable to stimulate G-CSF production. However, when gingival fibroblasts are stimulated with a combination of IL-4 and IL-1, greater than additive effects are observed in terms of the production of G-CSF. The results we have described here differ from those for murine 3T3 fibroblasts but are similar to those for human gingival fibroblasts. These studies substantiate the hypothesis that IL-4 may act indirectly to regulate myelopoiesis through its ability to promote G-CSF production by BMF stimulated with IL-1. It has been shown that IL-4 receptors are present on a variety of cells such as cloned stromal cell lines from bone marrow, fibroblasts, and other nonhematopoietic cells [23,24]. Furthermore, Northern blot analysis of RNA from IL-4-induced stromal cells indicates that IL-4 modestly increases the expression of tumor necrosis factor (TNF) [25–27]. These facts support the idea that BMF produce TNF after stimulation with IL-4 and that TNF acts synergistically with IL-1 to stimulate production of G-CSF. Apparently, the quantity of TNF produced is not adequate to stimulate the production of G-CSF by BMF, so that G-CSF may not be produced after stimulation with IL-4 alone. In the future, it will be necessary to determine the amount of TNF produced by BMF after stimulation by IL-4.

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